Expression Profile of MDM-2 Proteins in Chronic Lymphocytic Leukemia and Their Clinical Relevance

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The MDM-2 oncoprotein exists in an autoregulatory feedback loop with the tumor suppressor protein p53. Therefore, intracellular levels of these two proteins may play important roles in cell proliferation and tumorigenesis. Several MDM-2 proteins (Mr 35–100 Kd) have been demonstrated in human cell lines. We report here the expression profile of MDM-2 and p53 proteins in 87 cases of chronic lymphocytic leukemia (CLL) as detected by immunoblot analysis. The MDM-2 proteins (p57, p59, p67, and p90) were found to be overexpressed in different combinations in 56/87 (64%) of cases of CLL when compared with normal volunteers. The MDM-2 protein p57 was predominantly overexpressed 46/87 (53%) in CLL. In 22/87 (25%) cases of CLL p57 was overexpressed alone, and in 24/87 (28%) cases it was co-overexpressed with other MDM-2 proteins p59/p67/p90. Six of the 87 cases of CLL showed overexpression of the tumor suppressor protein p53 by immunoblot analysis, and five of those cases also co-overexpress MDM-2 protein p57. No statistically significant correlation of MDM-2 protein overexpression to clinical disease stage and history of previous chemotherapy of CLL patients has been found. However, considering the oncogenic potential of overexpressed MDM-2 proteins, a possible role of MDM-2 proteins in the promotion of CLL disease remains to be evaluated. Am. J. Hematol. 54:189–195

Key words: CLL; MDM-2 oncoprotein; prognosis

INTRODUCTION

Mutational inactivation of p53 tumor suppressor gene is the single most common genetic alteration in human cancer [1]. Several tumor virus proteins such as SV40 large T antigen, the adenovirus E1B, and the human papillomavirus E6 oncoproteins bind to wild-type p53 and inhibit its tumor-suppressor activity [2]. However, little is known about the cellular co-regulators that mediate p53 function or about their role in p53 inactivation and tumorigenesis. mdm-2 (murine double minute 2) gene has been proposed as a feedback regulator of p53 function by being transcriptionally induced by p53 and then inhibiting the p53 transactivation function by physically interacting with the amino terminal acidic activation domain of p53 [3–6].

The human MDM-2 gene (human analogue of mdm-2), which has been mapped to chromosome 12q13-14, was amplified in 30% of sarcomas along with overexpression of MDM-2 mRNA and protein [3,7]. Amplification of MDM-2 gene was implicated in tumor progression and metastasis in osteosarcoma, soft tissue sarcoma, and some malignant gliomas [3,7,8]. Several MDM-2 mRNAs and proteins (p90, p85, p76, p74, p58, and p57) have also been demonstrated in mouse and rat cell lines [9]. Expression of multiple MDM-2 proteins ranging from approximately 35 to 95 Kd and enhanced translation of MDM-2 mRNA have been demonstrated in choriocarcinoma cell lines [10]. Differential expression of a variety of MDM-2 mRNA and proteins (54–68 and 90–100 Kd, respectively) were detected in estrogen receptor negative and positive breast tumor cell lines [11,12]. However, in primary breast carcinomas we found overexpression of MDM2 proteins p57 and p90 irrespective of estrogen receptor status [13].

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Previous studies from our group showed overexpression of MDM-2 mRNA in different leukemias including 73% of chronic lymphocytic leukemias (CLLs), but no gene amplification was detected [14]. The present work was carried out to identify the expression profile of MDM-2 proteins in CLL cases and to study the possible relationship of these proteins to the clinical behavior of the disease. The data presented here showed that the p57/p59/p67/p90 MDM-2 proteins are overexpressed in 64% (56/87) of the CLL cases and are independent of clinical disease stage and history of previous chemotherapy.

MATERIALS AND METHODS

Specimen Collection

Peripheral blood specimens were obtained from patients evaluated in the leukemia clinic at The University of Texas M.D. Anderson Cancer Center during routine diagnostic/therapeutic procedures or from normal volunteers under approved protocols. The CLL samples used for analysis contained predominantly malignant cells. CLL cases having less than 15,000 lymphocyte/μl that co-express CD5 and CD19 were excluded from the study. Mononuclear cells were separated on Ficoll-Hypaque (Sigma Diagnostic, St. Louis, MO) and washed twice with phosphate-buffered saline (PBS). The cells were stored at −70°C until used for extraction of proteins. Extractions of protein under the conditions described below, immediately or following storage at −70°C for 1–4 weeks do not show any difference in the detection of MDM-2 proteins by immunoblot analysis.

Extraction of Protein

Cell pellets were lysed in TENN buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA [pH 8.0], 0.5% Nonidet P-40, 150 mM NaCl supplemented with 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml pepstatin) for 30 min on ice with frequent vortexing and then left on ice for 1 hr [15]. Lysates were clarified by microcentrifugation for 30 min at 15,000 rpm. Protein was estimated by the Bradford method, and 200 μg of the cell extract was run on a 9.5% SDS-PAGE gel and stained with Coomassie blue R-250 to check the protein profile and to see whether adjustment of protein concentration was needed.

Immunoblot Analysis of MDM-2 and p53 Proteins

Two hundred micrograms of cell extract from CLL patients and at least two normal volunteers was electrophoretically separated on a 9.5% SDS-PAGE gel and transferred to nitrocellulose paper. The nitrocellulose membrane was blocked with 5% non-fat milk in PBS containing 0.1% Tween 20 and 0.02% sodium azide for 6–8 hr at room temperature. The blot was then incubated for 4 hr with anti-MDM-2 polyclonal antibody 9326 (1:500 dilution with PBS containing 2.5% non-fat milk, 2.5% bovine serum albumin, and 0.1% Tween 20), which was raised by inoculating New Zealand white rabbit with a synthetic peptide P1 corresponding to 104–127 amino acids sequence of MDM-2 protein [3]. The membrane was washed with PBS containing 0.1% Tween 20. The blot was then incubated with 1:4,000 diluted anti-rabbit immunoglobulin linked to horseradish peroxidase (Amersham Life Science, Arlington Heights, IL) in PBS containing 1% non-fat milk and 0.1% Tween 20. Immunoreactive bands were developed using the ECL detection system (Amersham). The anti-MDM-2 monoclonal antibody IF-2 (Oncogene Science, San Diego, CA) was also used for immunoblot analysis to detect MDM-2 proteins at 2 μg/ml concentration in PBS containing 2.5% non-fat milk and 0.1% Tween 20 and incubated at room temperature for 6 hr. After the ECL detection, the membrane was stripped off the primary and secondary antibodies under the conditions recommended by Amersham Inc., and the stripped membrane was then blocked and probed with an anti p53 monoclonal antibody DO-1 (Oncogene Science). Overexpression of MDM-2 proteins in CLL samples was judged by comparing the expression level of MDM-2 proteins with the normal volunteers by densitometric scanning. Following immunoblot analysis each blot is stained with amido black solution (0.1% amido black in 45% methanol and 10% acetic acid) to check for equal loading of proteins in each lane.

RESULTS

Immunoblot Analyses of MDM-2 Proteins in Patients With CLL and in Normal Volunteers

Figure 1A is a representative immunoblot analysis showing overexpression of the MDM-2 proteins p57 and p59 in the lymphocytes of peripheral blood specimens from patients with CLL and normal volunteers. The arrows indicate the positions of MDM-2 proteins p57 and p59 with approximate molecular masses Mr 57,000 and 59,000, respectively. In Figure 1A cell extracts of CLL samples loaded in lanes 4, 7, and 11 show higher levels (4.3–4.9-fold) and lanes 3, 5, 9, 10, and 13 show intermediate levels (2–2.9-fold) of overexpression of MDM-2 protein p57 when compared with the p57 protein in normal volunteers (lanes 14 and 15). CLL samples in lanes 1 (2.2-fold), 6 (4.2-fold), and 10 (3.2-fold), showed overexpression of MDM-2 protein p59 when compared with the level of p59 protein in control samples (lanes 14, 15). Mononuclear cells from thirty normal adult volunteers (of both sexes) of ages between 20–70 were also analyzed by immunoblot analysis. The immunoblot showed

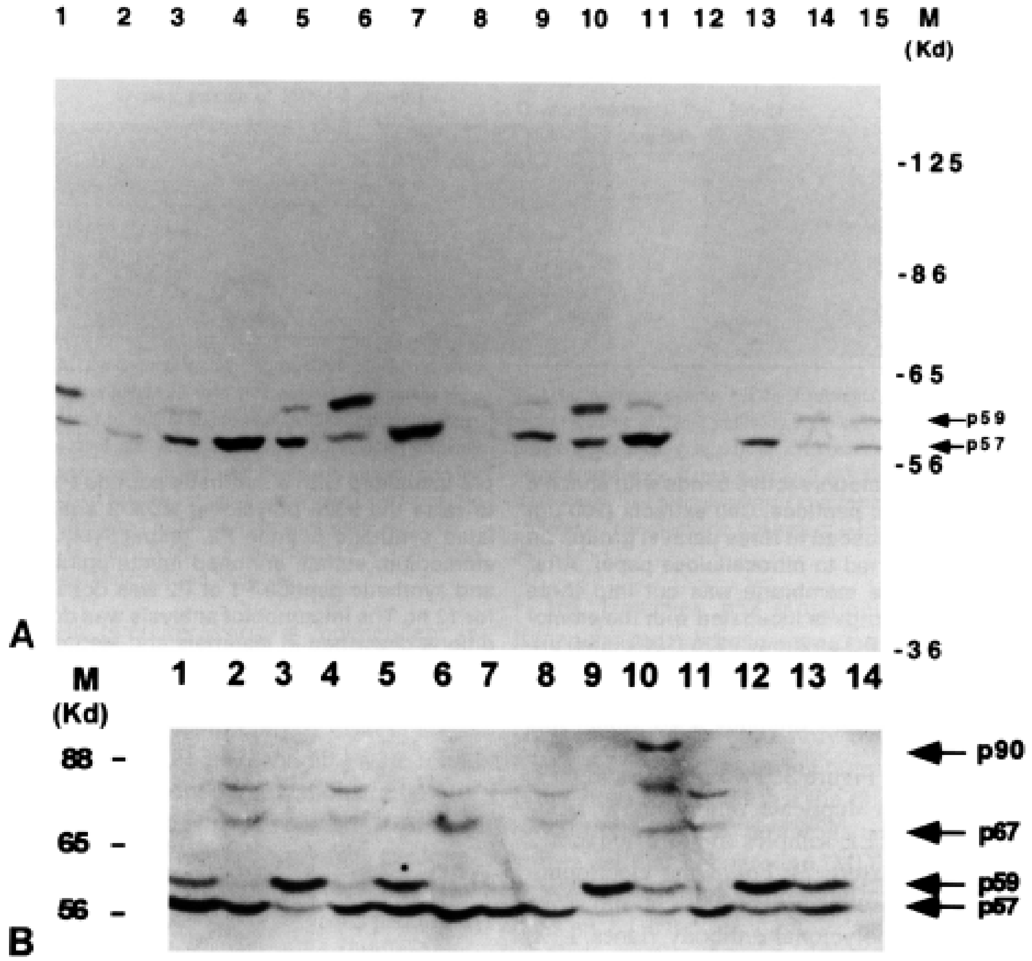

Fig. 1. Immunoblot analyses of leukemia samples. Two hundred micrograms of mononuclear lymphocyte cell extract of patients with CLL and normal volunteers was subjected to immunoblot analysis using MDM-2 polyclonal antibody 9326 (1:500 dilution) and immunoreactive bands were developed by chemiluminescence as described in Materials and Methods. A, lanes 1–13 show samples from patients with CLL and lanes 14 and 15 show samples from normal volunteers; B, lanes 1–13 represent samples of CLL patients and lane 14 a sample from a normal volunteer. M, molecular weight standards in Kd.

trace amounts of p57 and p59 MDM-2 proteins in these samples (data not shown).

Figure 1B is a representative immunoblot showing expression of different MDM-2 proteins including p57, p59, p67, and p90. Lanes 2 and 6 show overexpression of p67 and p57. Lane 10 shows expression of p90 along with p57 and p59. A trace amount of p74 protein is also detected in some CLL samples (see lanes 2, 4, 6, 8, 10, 11). Similarly, some normal volunteers also showed a low level of p74 MDM-2 protein expression, which suggested that expression of p74 MDM-2 protein may not be specific for CLL and therefore we excluded the p74 expression profile from this study.

Figure 2A shows a representative immunoblot analysis with samples expressing MDM-2 proteins p57, p59, p67, and p90 when incubated with the MDM-2 polyclonal antibody 9326 enriched by ammonium sulfate fractionation. Parallel blots were incubated with the enriched MDM-2 antibody 9326 pre-incubated with a synthetic peptide P1 (YRNLVVVNQQESSDSGTSVSENRC) (Fig. 2B) used to raise the 9326 antibody or an unrelated synthetic peptide P2 (SEDYSLSEEGQELSDED-DEVYQVT) (Fig. 2C). The P1 peptide competed off the immunoreactive bands p57, p59, p67, and p90 (Fig. 2B). However, under similar incubation conditions no competition of the immunoreactive bands p57, p59, p67, and p90 was observed with the unrelated synthetic peptide P2 (Fig. 2C). This specific competition of the immunoreactive bands with the peptide P1 (which was used for raising the polyclonal Ab 9326) suggests that the antibody 9326 reacts specifically with MDM-2 protein harboring the amino acids sequence between 104–127 (P1 peptide).

A comparison of immunoreactive bands detected using polyclonal antibody 9326 and the IF2 monoclonal

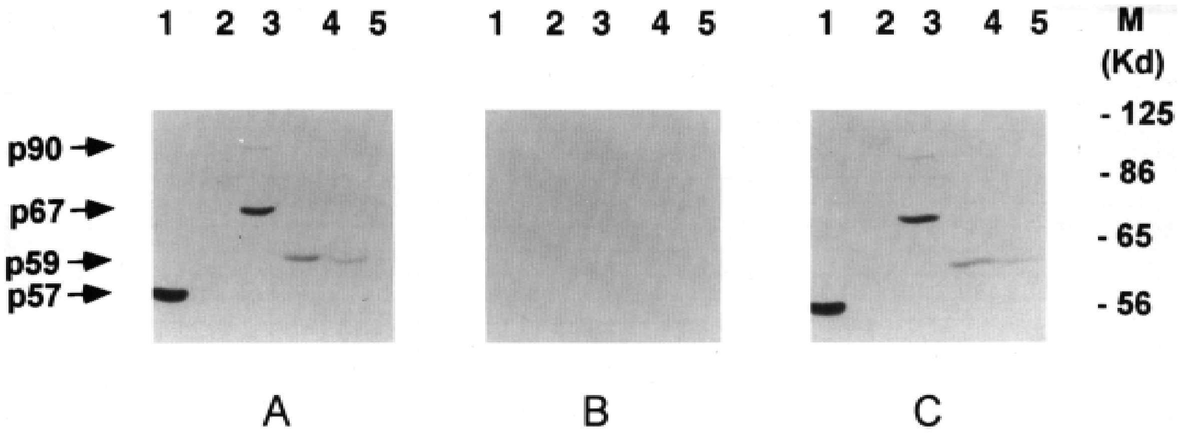

Fig. 2. Competition of immunoreactive bands with specific and non-specific synthetic peptides. Cell extracts (200 µg) from 5 CLL patients were loaded in three parallel groups on the same gel and transferred to nitrocellulose paper. After transfer the nitrocellulose membrane was cut into three strips. In A one of these strips is incubated with the ammonium sulfate enriched MDM-2 antibody 9326 (100 µg/ml) under the conditions described in the legend to Figure 1 and Materials and Methods. In B and C parallel blots were incubated with the enriched MDM-2 antibody 9326, which was pre-incubated with a synthetic peptide P1 (which was used to raise the 9326 polyclonal MDM-2 antibody) or an unrelated synthetic peptide P2, respectively. Preincubation of ammonium sulfate enriched immunoglobulin of 9326 sera and synthetic peptide P1 or P2 was done in 1:1 molar ratio for 12 hr. The immunoblot analysis was done under the conditions described in Materials and Methods and in the legend to Figure 1. The molecular weight markers are shown on the right-hand side of the figure.

antibodies was shown in Figure 3. Two mixtures of CLL samples were loaded in duplicate onto a 9.5% SDS-PAGE (lanes 1 and 3, CLL samples mixture 1; lanes 2 and 4, CLL samples mixture 2). Following electrophoretic transfer nitrocellulose membrane was cut into strips and probed with 9326 polyclonal antibody (lanes 1, 2) and IF2 monoclonal antibody (lanes 3, 4). Both the antibodies detected p57, p67, and p90 proteins. However, the p59 immunoreactive band was detected by 9326 antibody only (see lanes 1 and 2). The immunoreactive bands detected following ECL treatments require short exposure (15 sec) when 9326 antibody was used as a primary antibody, whereas long exposure (1 hr) was required for detecting immunoreactive bands when IF-2 antibody was used as a primary antibody. We did not detect any immunoreactive bands in 15-sec exposure when IF2 was used as the primary antibody. This difference in exposure time suggests that 9326 is a relatively stronger antibody than IF2 in detecting MDM-2 proteins under the conditions used for immunoblot analysis. The 9326 and IF2 antibodies detect similar bands (p57, p67, and p90) except p59.

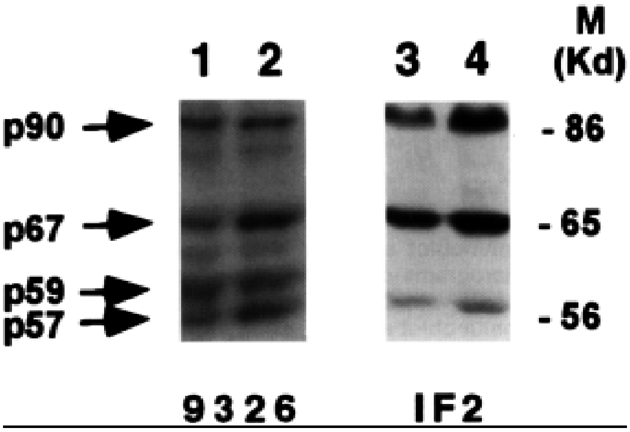

Fig. 3. Comparison of immunoblot analysis using polyclonal antibody 9326 and monoclonal antibody IF2. Two hundred micrograms of CLL sample mixture 1 (lanes 1, 3) and CLL sample mixture 2 (lanes 2, 4) was electrophoresed in a 9.5% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was cut into strips blocked in 5% non-fat milk and incubated with primary antibodies 9326 (lanes 1, 2) and IF2 (lanes 3, 4). The membranes probed with 9326 and IF2 antibody were exposed for 15 sec and 1 hr, respectively, following ECL treatment. The immunoblot analysis was done as described in Materials and Methods and in the legend to Figure 1.

Relationship of MDM-2 Protein Overexpression, Previous Chemotherapy, and Clinical Staging of CLL

Table I summarizes the relationship between MDM-2 protein overexpression and clinical disease stage of patients who had or did not have previous chemotherapy. In stages A the percentage of CLL cases overexpressing MDM-2 proteins is between 57–60% irrespective of previous history of chemotherapy ($P = 0.83$). In clinical stages B and C the number of patients overexpressing MDM-2 proteins were between 77–80% ($P = 0.86$) and 57–67% ($P = 0.68$), respectively, and similarly independent of previous chemotherapy. These results suggest

TABLE I. MDM-2 Protein Overexpression, Disease Stage, and History of Previous Chemotherapy*

Staging (Binet)	Overexpression of MDM-2 proteins [No. of cases (%)] No prior chemotherapy	Prior chemotherapy
A	17/30 (57)	9/15 (60)
B	8/10 (80)	10/13 (77)
C	4/7 (57)	8/12 (67)
Total	29/47 (62)	27/40 (68)

*Patients who express MDM-2 proteins at intermediate (2–3-fold over normal volunteers) and higher level (>3-fold over normal volunteers) were considered as overexpressors of MDM-2 proteins. MDM-2 proteins were detected by immunoblot analysis using a MDM-2 polyclonal antibody 9326. The fold overexpression was determined by densitometric scanning of the corresponding bands of patients' samples and normal volunteers. No statistically significant differences in MDM-2 proteins overexpression in CLL disease stage A ($P = 0.83$), B ($P = 0.86$), and C ($P = 0.68$) have been found. For details see Materials and Methods.

that MDM-2 protein overexpression is independent of clinical disease stage and prior chemotherapy.

Table II summarizes the possible relationship of overexpression of MDM-2 proteins to clinical disease stage. The MDM-2 proteins p57, p59, p67, and p90 were found to be overexpressed in different combinations in 64% of CLL cases when compared to normal volunteers. Among the MDM-2 proteins the p57 appeared to be the predominantly overexpressed (53%) protein. No clear relationship of overexpression of a particular MDM-2 protein to a clinical disease stage has been observed (clinical disease stage A, $P = 0.68$; B, $P = 0.48$; C, $P = 0.79$). The source of patients' samples used in this study was the same for Tables I and II.

Overexpression of Tumor Suppressor Protein p53 in Patients With CLL

Immunoblot analysis for tumor-suppressor protein p53 was performed on all the Western blots used for detecting MDM-2 proteins. Following stripping, the membranes were reprobed with the anti-p53 antibody DO-1. Figure 4 shows a representative immunoblot assay using anti-p53 antibody DO-1. In Figure 4, lanes 1, 4, and 5 showed a high level of expression of p53 protein. No expression of p53 protein was detected in normal volunteers (see lanes 13 and 14). In 6 out of 87 CLL cases we detected high level of p53 protein expression by immunoblot analysis. Five out of these six cases also overexpressed the MDM-2 protein p57. Among these five CLL cases one is in clinical disease stage A, two in stage B, and two in stage C. All five patients are alive with medium follow-up of 21 months.

DISCUSSION

Mutational inactivation of tumor suppressor protein p53 has been detected in a wide variety of human cancers

TABLE II. Relationship of Overexpression of MDM-2 Proteins p57, p59, p67, p76, and p90 and Disease Stage of CLL*

Overexpression of MDM-2 proteins	No. of cases	Disease stage, no. of cases (%) A	B	C
—	31	19 (61)	5 (16)	7 (23)
p57	22	9 (41)	8 (36)	5 (23)
p59	10	5 (50)	2 (20)	3 (30)
p57 and p59	9	3 (33)	4 (44)	2 (22)
p57 and p90	8	4 (50)	2 (25)	2 (25)
p57 and p67	7	5 (71)	2 (29)	0 (0)
Total	56	26 (46)	18 (32)	12 (21)

*—, no overexpression. MDM-2 proteins were detected by immunoblot analysis using MDM-2 antibody 9326 as described in Materials and Methods. No statistically significant differences in the distribution of MDM-2 proteins to disease stages A ($P = 0.48$), B ($P = 0.47$), and C ($P = 0.79$) were found. The patients who are considered as overexpressors of MDM-2 proteins by densitometric scanning of the immunoblot bands are the same as in Table I.

[1,16]. However, p53 mutations in hematological malignancies are relatively rare compared with those seen in other neoplastic diseases [17]. Wild-type p53 protein has been suggested to play an important role in hematopoietic cell maturation, possibly by inhibiting the cell proliferation that occurs during terminal differentiation [18]. It has been suggested that the tumor suppressor function of wild-type p53 can be abrogated by physical interaction with a cellular oncoprotein, MDM-2 [4–6].

We analyzed the expression profiles of MDM-2 proteins in 87 cases of CLL in an effort to delineate the possible relationship of MDM-2 protein expression to clinical disease stage of chronic lymphocytic leukemia. The data presented here showed that four MDM-2 proteins (p57, p59, p67, and p90) in different combinations were overexpressed in 64% of patients with CLL when compared to normal volunteers. This detection of MDM-2 protein overexpression by immunoblot analysis is comparable to our earlier reports and reports from two other groups regarding overexpression of MDM-2 mRNA in different leukemias including CLL [14,19,20].

We also detected high levels of p53 protein expression in 6/87 (7%) patients with CLL. When a high level of p53 expression is detected, it is believed to represent a missense mutation of the p53 gene [18,21–24]. The mutation increases the half-life of wild type p53 protein and allows its detection on immunochemical analyses [18,21–24]. However, an extended half life (>2 hr) of wild type p53 in human breast carcinoma cell lines has also been demonstrated [12]. Five of these six cases that overexpress p53 protein showed concomitant overexpression of MDM-2 protein p57, which suggests that abnormality in p53 expression could co-exist with abnormality in MDM-2 expression. Out of these five CLL patients one was in clinical stage A, two were in stage B, and two were in stage C. Similar overexpression of both the p53 and MDM-2 proteins was detected in soft tissue

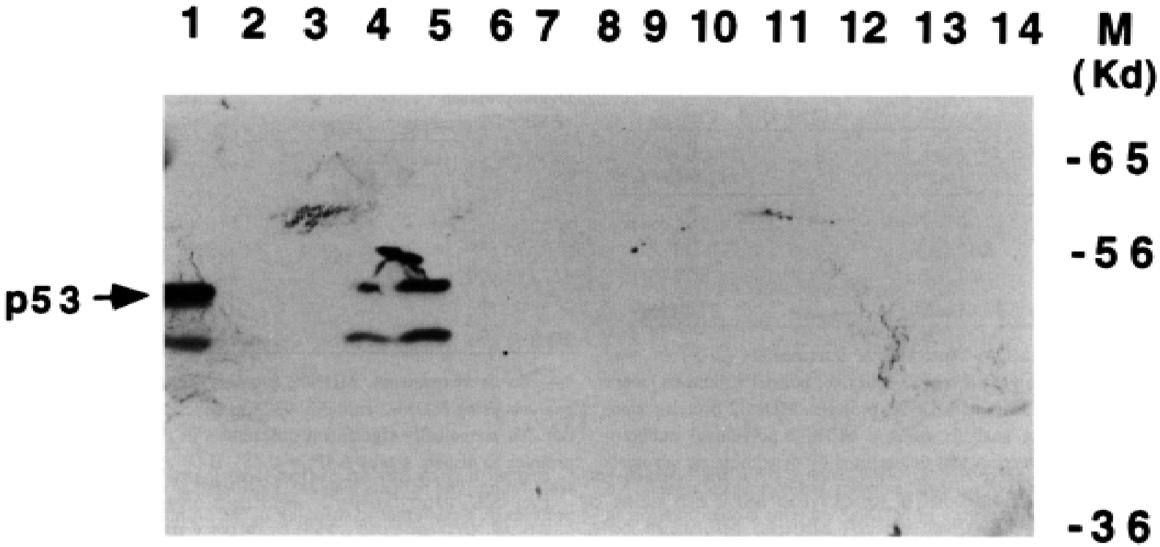

Fig. 4. Immunoblot analyses of tumor suppressor protein p53. Immunoblot analyses were done as described in the legend to Figure 1 and Materials and Methods using anti p53 monoclonal antibody DO-1 at a 2 μg/ml concentration. Lanes 1–12 are cell extracts from patient with CLL and lanes 13 and 14 are cell extracts from normal volunteers.

sarcoma and was found to be associated with poor clinical prognosis [25]. In two choriocarcinoma cell lines (JAR and JEG-3) as well as in breast MCF7 cell line, overexpression of both wild-type p53 and MDM-2 proteins has been reported [10–12].

Our results derived from immunoblot analysis of MDM-2 proteins differ from those reported recently by Watanabe et al. [19] based on messenger RNA analysis and immunohistochemistry which suggested a possible association of MDM-2 protein overexpression to advance disease stage (stage C) in patients with CLL. The observation of Watanabe et al. [19] was based on only 17 B-CLL cases which may not be an adequate representation of the disease.

The lack of association of MDM-2 overexpression to poor prognostic factors such as clinical stage or prior chemotherapy does not support an important role for MDM-2 in the progression of the CLL disease as reported earlier [19]. However, considering the oncogeneic potential of overexpressed MDM-2 proteins [3,7,8] a possible role in promotion of the CLL disease can not be overruled.

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